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Protoplast culture and somaclonal variability of species of series *Juglandifolia*

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Abstract

Mesophyll protoplasts of species of series *Juglandifolia (Solanum rickii, S. lycopersicoides, S. ochranthum* and *S. juglandifolium*) were isolated and cultured in liquid nutrient media TM-2 or KM8P. The cell colonies formed were transferred onto agar-solidified media TM-3 or GM, and 10 to 15 days later onto TM-4, PRM, MS3ZG, KK or C regeneration media. Formation of the shoots for S. *rickii* and *S. lycopersicoides* was observed after 30 to 35 days on regeneration medium. The regenerated shoots were rooted on hormone-free MS medium. Morphological and cytogenetic analyses have shown that somaclonal variants might arise in the course of plant regeneration from protoplasts of these species.

Abbreviations: BAP – 6-benzylaminopurine; 2, 4-D – 2, 4 dichlorophenoxyacetic acid; NAA – α-naphthaleneacetic acid; GA_3 – gibberellic acid; IAA – indole-3-acetic acid; MS – Murashige and Skoog medium; B5 – Gamborg medium; TRIS – tris(hydroxymethyl) aminomethane

Introduction

According to morphological and cytogenetic traits *Solanum lycopersicoides* (woody shrub), *Solanum rickii* (perennial herb), *Solanum ochranthum* and *Solanum juglandifolium* (woody lianas) four South American wild species of section *Petota*, subsection *Potatoe* are classified into the series *Juglandifolia*. Based upon morphological differences they are subdivided into two groups: *juglandifolium-ochranthum* and *lycopersicoides-rickii*. The species of the series *Juglandifolia* (Solanaceae) occupy an intermediate taxonomic position between *Solanum* and *Lycopersicon*. Morphology of their anthers is typical of *Solanum*. They are not coalescent, dehisce terminally and their pollen is fertile. Due to these traits they are related to *Solanum*. However, according to other morphological, cytogenetic and biochemical traits, as well as crossing characteristics, the species of series *Juglandifolia* are close to *Lycopersicon*. For example, they have a yellow corolla, and pinnatisected leaves and they do not form tubers or stolons. There are diploid $2n = 2x = 24$ (Rick, 1979). Moreover the restriction patterns of chloroplast DNA of these non-tuberous *Solanum* species' especially of *S.lycopersicoides*, resemble those of *Lycopersicon* than *Solanum* (Hosaka *et al.*, 1984). The species of series *Juglandifolia* are insect resistant. Furthermore *S. lycopersicoides* and *S rickii* are low temperature tolerant. *S. lycopersicoides* is resistant to virus diseases and *Botrytis* rot. *S.juglandifolium* and *S. ochranthum* tolerate water logging and are resistant to root diseases (Rick, 1987). Therefore the species of series *Juglandifolia* are considered to be good sources of valuable agronomic traits. However, sexual crosses between these species and cultivated tomato are limited due to bilateral or unilateral incompatibility. The species of *Juglandifolia* series do not cross with other species of

Juglandifolia series do not cross with other species of section *Petota*, though fertile sexual hybrids between *S. lycopersicoides* and *L. esculentum* have been obtained (Rick *et al.*, 1986; Vorobjeva *et al.*, 1992). *S. rickii* can cross only with *S. lycopersicoides*, *S. ochranthum* and *S. juglandifolium* failed to cross in any combination tested (Rick, 1979).

Overcoming crossing barriers and introgression of genes from wild species into cultivated tomato have become possible with the development of the methods of protoplast isolation, culture and fusion. The first successful cultivation of protoplasts and regeneration of the plants for the species of series *Juglandifolia* were reported by Handley & Sink (1985b) and Tan *et al.* (1987) using suspension culture-derived and mesophyll protoplasts of *S. lycopersicoides*. Somatic hybrids between *S. lycopersicoides* and *L. esculentum* have also been obtained (Handley *et al.*, 1986; Tan, 1987). *S. rickii*, which is morphologically similar to *S. lycopersicoides*, was also used for production of somatic hybrids with *L. esculentum* (O'Connell & Hanson, 1986). Conditions for protoplast cultivation and plant regeneration has been elaborated for many Solanaceae species but only for *S. lycopericoides* from series *Juglandifolia*. Gavrilenko & Piven (1990) managed to obtain callus colonies from mesophyll protoplasts of *S. ochranthum*. Data on plant regeneration or successful protoplast cultivation for *S. rickii* and *S. juglandifolium* are absent.

In this paper the results of experiments on protoplast isolation, culture as well as plant regeneration for species of series *Juglandifolia* are presented. Genetic stability of *S. lycopersicoides* and *S. rickii* was also studied in the course of *in vitro* 'plant-protoplast-plant' cycle.

Materials and methods

Plant material

Seeds of *Solanum lycopersicoides* Dun. (LA 2407), *S. rickii* Corr. [LA 1974, syn. *S. sitiens* (Marticorena & Quezada, 1977)], *S. ochranthum* Dun. (LA 2682) and *S. juglandifolium* Dun. (LA 2788) were a generous gift from Prof. C.M.Rick (Tomato Genetics Stock Center, Davis, Calif. USA). They were sterilized sequentually in 70% ethanol (30–40 s), 3% sodium hypochlorite (6–8 min), washed in sterile distilled water and, following scarification, germinated on wet filter paper in Petri dishes. After 8 to 10 days, the plantlets were transferred to half-strength hormone-free MS medium (Murashige & Skoog, 1962) containing 1.0 mg l^{-1} thiamine-HCl, 100 mg l^{-1} myo-inositol, 20 g l^{-1} sucrose as well as 8.0 g l^{-1} agar (Serva) and were grown at 25 °C, illumination at 2000–3000 lux under a 16-h photoperiod. Plants were propagated by cuttings. The population of plants for each species was derived from a single seed.

Culture media

For plant regeneration from mesophyll protoplasts the following media were tested: for protoplast culture - W-S-S (Sidorov *et al.*, 1984), KM8P (Kao & Michayluk, 1975), TM–2 (Shahin, 1985); for minicallus formation - GM (Tan *et al.*, 1987), TM-3 (Shahin, 1985); and for morphogenesis - TM-4 (Shahin, 1985), MS3ZG (Handley & Sink, 1985a), PRM (Ratushnyak *et al.*, 1989), C (Shepard & Totten, 1977) and Koblitz & Koblitz (1982) (hereafter KK) which consisted of mineral salts of MS medium, vitamins of B5 medium (Gamborg *et al.*, 1968), 0.2 mg l^{-1} IAA and 2 mg l^{-1} BAP. The pH of the media was adjusted by 0.1N KOH, then agar (Serva) was added, and autoclaved for 15 min at 125 °C. The enzyme mixture and the medium for protoplast culture were filter sterilized.

Protoplast isolation

Approximately 1 g leaves of 18 to 20 day-old aseptically grown plants of *S. rickii* were cut into thin strips and placed into 5 ml of enzyme mixture I containing 0.2% cellulase 'Onozuka R-10' (Serva), 0.1% driselase (Sigma), 0.5 M sucrose and 5 mM $CaCl_2$, pH= 5.6. Enzymatic treatment was carried out in glass Petri dishes (60 mm diam) for 14–16 h in the darkness at 25 °C. The enzyme mixture containing the protoplasts was filtered through a nylon screen (80 μm pore size) and centrifuged for 3 min at 100 × *g*. Floating protoplasts were collected with a Pasteur pipette and washed twice with W5 medium (Medgyesy *et al.*, 1980) by centrifugation for 2 min at 60 × *g*. Mesophyll protoplasts of *S. lycopersicoides*, *S. ochranthum* and *S. juglandifolium* were isolated from 3-week-old plants following treatment in enzyme solution II according to Tan *et al.* (1987). Protoplast density was estimated using a hemocytometer.

Protoplast culture and plant regeneration

The protoplasts were placed in 90 mm plastic Petri dishes containing 5 ml corresponding liquid media W-S-S (2 mg l^{-1} NAA, 0.2 mg l^{-1} 2, 4-D and 0.5 mg l^{-1} BAP), TM-2 (0.5 mg l^{-1} Zea and 1 mg l^{-1} NAA) or KM8P (1 mg l^{-1} NAA, 0.2 mg l^{-1} 2, 4-D and 0.5 mg l^{-1} Zea) and were cultured during the first five days in darkness at 25 °C, and then under 600 lux illumination, 16-h photoperiod and 25 ± 1 °C. The plating efficiency of protoplasts was determined 2–3 weeks after isolation as the ratio of the number of dividing cells to the total number of initial protoplasts multiplied by 100. Minicalli formed on TM-3 (0.5 mg l^{-1} BAP and 0.2 mg l^{-1} 2, 4-D) or GM (0.5 mg l^{-1} BAP and 0.05 mg l^{-1} NAA) agar solidified media were transferred to TM-4 (1 mg l^{-1} Zea and 0.2 mg l^{-1} GA_3), MS3ZG (3 mg l^{-1} Zea and 0.1 mg l^{-1} GA_3), PRM (2.5 mg l^{-1} BAP and 0.2 mg l^{-1} IAA), KK or C (0.5 mg l^{-1} BAP and 0.1 mg l^{-1} NAA) regeneration media. The regenerating shoots were rooted on hormone-free MS medium 55 to 65 days after protoplast isolation. The morphogenetic index was determinated as a ratio of the number of calli capable of regeneration to the total number of calluses multiplied by 100 (minicallus formation index and index of rooted shoots were determinated in a similar way). Formation of minicallus, morphogenesis and rooting of shoots were successful under the conditions of a 16-h photoperiod (fluorescent lamps, 3000 to 4000 lux) at 25 ±1°C.

Chromosome counting

Preparations of metaphase mitotic chromosomes from root tips of *in vitro* clones were used for cytological studies. The root tips were treated in an aqueous α-monobromonaphthalene saturated solution, fixed in a 1 : 3 mixture of acetic acid : ethanol, stained in 1% acetoorcein and squashed in a drop of lactic acid.

Isozyme analysis

Proteins were isolated from young leaves in buffer solution containing 0.05 M TRIS, 0.01 M β - mercaptoethanol, 12% glycerol, pH = 7.5 (leaf : buffer - 1 : 2). Electrophoretic separation of proteins was carried out in 10% polyacrylamide gels. Electrode buffer used was veronal one (5.5 g l^{-1} veronal and 1 g l^{-1} TRIS). For visualization of multiple molecular forms of esterase and glutamate oxaloacetate transaminase the gels were treated according to Brewer (1970).

Results and discussion

Protoplast isolation and culture

After treatment of *S. rickii* leaves with enzyme solution I, 6 × 10^6 protoplasts were isolated from 1 g of leaf tissue. The same enzyme solution was unsuitable for *S. lycopersicoides, S. ochranthum* and *S. juglandifolium*. Enzyme solution II which was used by Tan *et al.* (1987) for *S. lycopersicoides* was better for these species. Approximately 2–4 ×10^4 – 10^5 protoplasts were obtained, after incubation of 1 g of *S. lycopersicoides, S. ochranthum* or *S. juglandifolium* leaves in enzyme solution II for 14–16 h.

The isolated protoplasts of *S. rickii* (Fig. 1A), *S. lycopersicoides* and *S. juglandifolium* were cultivated in TM-2 medium. The plating density was 1-3 × 10^5 protoplasts per ml. Resynthesis of cell wall proceeded on the third or fourth day after isolation. The first cell divisions were observed on the fifth to eighth day (Fig. 1B). Plating efficiency of protoplasts for *S. lycopersicoides* and *S. rickii* reached 6%, and 3% for *S. juglandifolium* (Table 1). SCM medium used for cultivation of *S. lycopersicoides* protoplasts by Tan *et al.* (1987) appeared to be more suitable than TM-2. The plating efficiency on this medium reached 31%.

W-S-S and TM-2 media were to be unsuitable for cultivation of *S. ochranthum* protoplasts. The protoplasts started resynthesis of their cell walls on the second or third day in KM8P medium. The first cell divisions were observed on the fifth day. The plating efficiency reached 3% (Table 1).

After 16 to 20 days of protoplast culture, minicolonies of *S. rickii* (up to 1 mm diam, Fig. 1C) were transferred to TM-3 medium for further minicallus formation. GM medium was used for minicallus formation from *S. lycopersicoides, S. ochranthum* and *S. juglandifolium* minicolonies. The cell colonies grew to 2 to 5 mm diam and became green-coloured on these culture media (Fig.1D).

Plant regeneration

Minicalluses of *S. rickii* after 10 to 16 days growth on TM-3 medium were transferred onto TM-4 or PRM regeneration media to induce organogenesis. However, plant regeneration was observed only on PRM medium. Minicalluses grew well on this medium and became dark-green and denser. A month later, after adventive bud formation, they were transferred to fresh PRM nutrient medium. Calluses regenerated in sepa-

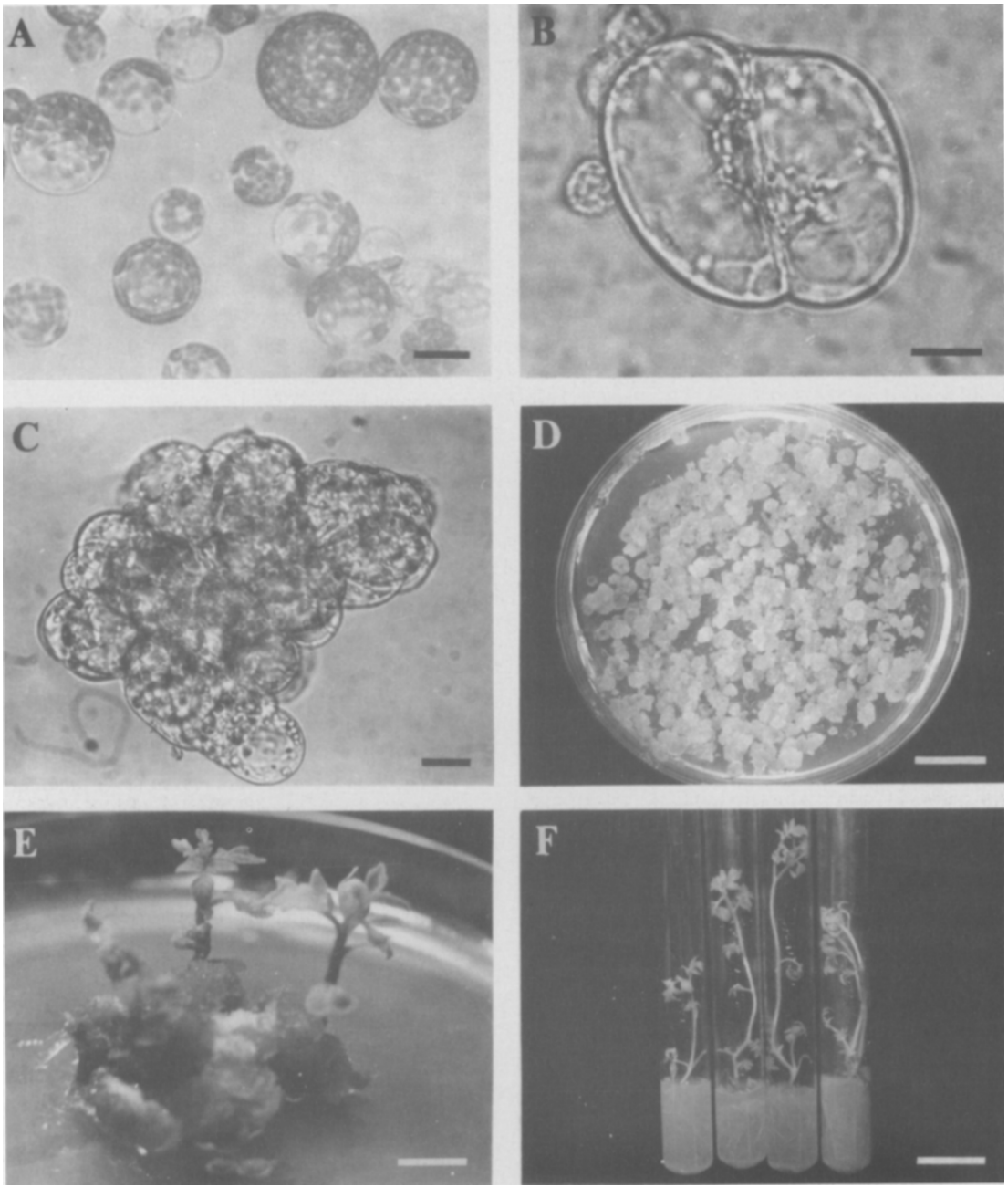

Fig. 1. Shoot regeneration from leaf mesophyll protoplasts of *Solanum rickii*. (*A*) Leaf mesophyll protoplasts. (*B*) First cell division. (*C*) Cell colony from single protoplast. (*D*) Cell colonies on TM-3 medium. (*E*) Shoot regeneration from cell colonies. (*F*) Rooted plants. Bars represent 45 μm (A), 32 μm (B), 1 μm (C), 15 mm (D), 5 mm (E), 23 mm (F).

rate shoots bearing two or three leaves (Fig. 1E). The regeneration index was 80% (Table 1).

Shoot regeneration for *S. lycopersicoides* proceeded on MS3ZG, KK and PRM media. Adventive buds which gave rise to shoots appeared a month after transfer of minicalli to these regeneration media. Regeneration index for *S. lycopersicoides* on MS3ZG and KK media was similar (Table 1) and approached that reported by Tan *et al.* (1987). Regeneration on PRM medium differed from that on MS3ZG or KK media.

Table 1. Stages of mesophyll protoplast cultivation of the species of series *Juglandifolia*.

Species	Stages	Media used	Duration of cultivation (days)	%[a]
S. ochranthum	Cell colony formation	KM8P	16–20	3
	Minicalli formation	GM	12–17	86
S. juglandifolium	Cell colony formation	TM-2	16–20	3
	Minicalli formation	GM	12–17	88
S. lycopersicoides	Cell colony formation	TM-2	16–20	6
	Minicalli formation	GM	10–16	93
	Regeneration of shoots	MS3ZG	29–30	85
		KK	29–30	85
		PRM	45–60	66
	Rooting of shoots	MS	10–15	46
S. rickii	Cell colony formation	TM-2	16–20	6
	Minicalli formation	TM-3	10–16	97
	Regeneration of shoots	PRM	29–30	80
	Rooting of shoots	MS	10–15	58

[a] These data were calculated for three independent experiments

Calluses were smaller in size on PRM medium and light-green. The period from transferring minicalluses onto this regeneration medium to the first shoot formation was longer, 1.5 to 2 months (Table 1). Regeneration index was also lower than that for MS3ZG or KK media (Table 1).

Regenerated shoots of *S. rickii* and *S. lycopersicoides* were rooted on hormone-free MS medium (Fig. 1F). Rooting efficiency was approximately the same for both species (Table 1).

Several media were tested for *S. juglandifolium* shoot regeneration. However, after transfer to TM-4, MS3ZG, PRM, KK and C media, almost all calluses protoclones died on the tenth to fourteenth day. Only a few calluses proceeded to grow on PRM medium and became dense and dark-green coloured. In that state they were alive for several months but showed no sign of regeneration.

TM-4, MS3ZG and PRM media were tested for *S. ochranthum* plant regeneration but all minicalluses died within 2 to 3 weeks after transfer to these regeneration media.

Analysis of regenerated plants

Morphology of the regenerants was estimated in the greenhouse using cleft grafting of *S. lycopersicoides* and *S. rickii* onto *L. esculentum* cvs. De Baraou, Delicious and Rutgers. Without grafting rooting of these species or their protoclones was practically impossible. Shoots from six independent protoclones for each species and their initial parental forms were studied. Two protoclones of *S. lycopersicoides* (7L, 2L) and three of *S. rickii* (2R, 4R and 5R) differed from the parental plants by leaf shape, size (Fig. 2 Al–A3 and B1–B3) and hairiness.

Cytogenetic analysis showed that these protoclones differed from initial parental plants in chromosome number (Fig. 2 C1–C3). Two of seven *S. lycopersicoides* protoclones and four of seven *S. rickii* protoclones were tetraploid $2n = 4x = 48$ (Table 2).

Analysis of multiple molecular forms of esterase and glutamate oxaloacetate transaminase of *S. rickii* and *S. lycopersicoides* showed that all somaclones had identical zymograms as their parents.

Somaclonal variability for the series of *Juglandifolia* regenerated from leaf or stem explants, callus, suspension culture, or protoplasts has not been previously reported (Kut & Evans, 1982; Handley & Sink,

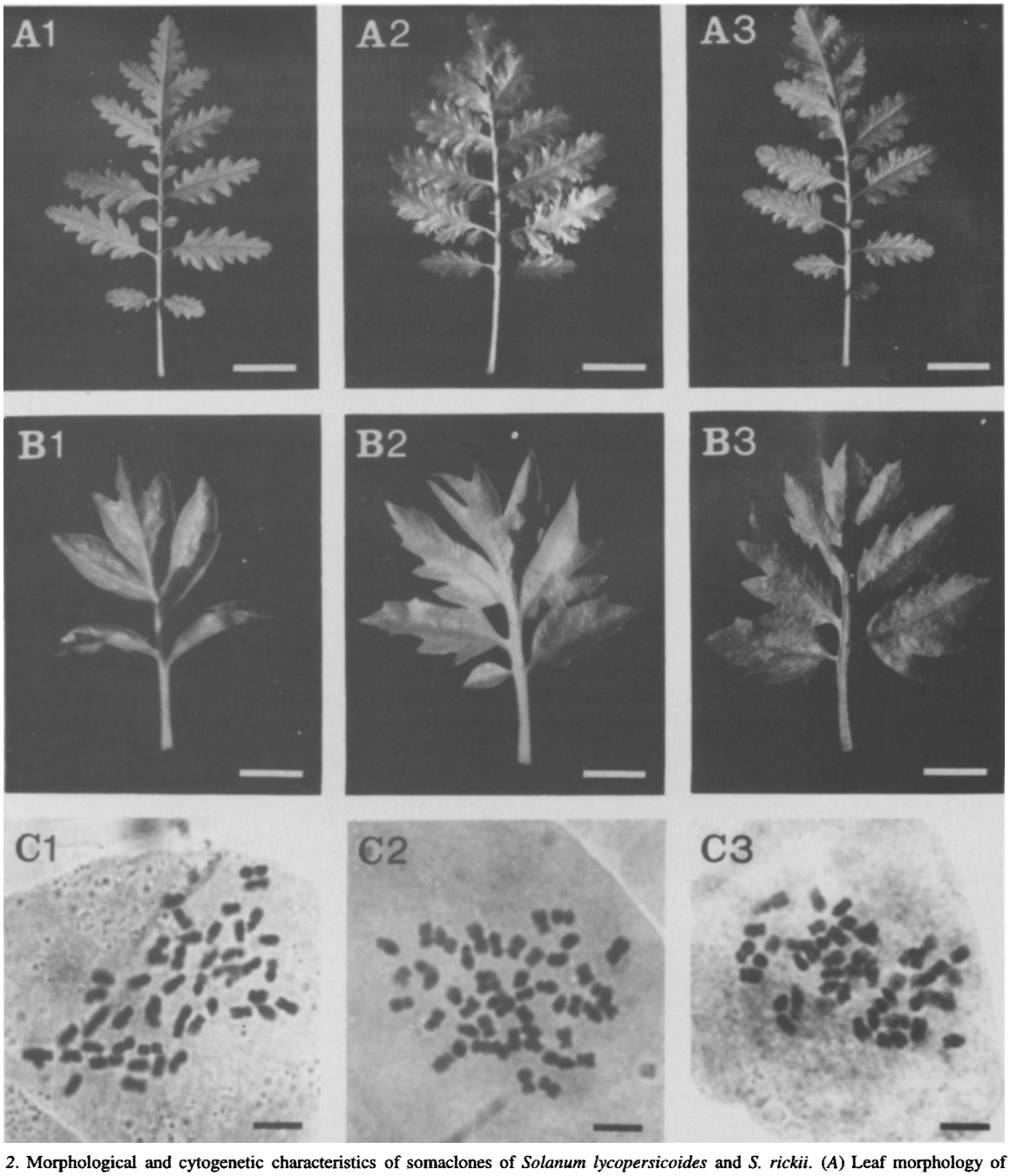

Fig. 2. Morphological and cytogenetic characteristics of somaclones of *Solanum lycopersicoides* and *S. rickii*. (*A*) Leaf morphology of *S. lycopersicoides* (1 – initial form, 2 – somaclone 12L, 3 – somaclone 7L). (*B*) Leaf morphology of *S. rickii* (1 – initial form, 2 – somaclone 4R, 3 – somaclone 5R). (*C*) Metaphase plates of *S. rickii* (1 – somaclone 4R, 2 – somaclone 5R, $2n = 4x = 48$) and *S. lycopersicoides* (3 – somaclone 7L, $2n = 4x = 48$). Bars represent 17 mm (A1, A2 and A3), 11 mm (B1), 12 mm (B2), 14 mm (B3), and 40 μm (C1, C2 and C3).

1985a, b; Tan *et al*., 1987; Gavrilenko & Piven, 1990). But genetic instability has come to be expected among regenerants of other Solanaceae, especially tomato, potato and tobacco (Evans *et al*., 1984; Ramulu, 1991 and references therein). For *L. esculentum* somaclones with alterations in form and colour of leaves, flowers, fruit and ploidy levels were found (Evans *et al*., 1984) as well as mutants resistant to *Fusarium oxyspo-*

Table 2. Cytogenetic characteristics of *Solanum rickii, S. lycopersicoides* protoclones and their initial plants.

Genotype	Number of roots studied	Number of metaphase plates studied	Number of chromosomes
S. lycopersicoides (LA 2407)	180	74	24
1L	141	51	24
3L	178	67	24
5L	205	94	24
8L	197	82	24
7L	217	103	48
9L	163	58	24
12L	245	114	48
S. rickii (LA 1974)	263	107	24
2R	136	132	48
4R	157	154	48
5R	192	187	48
9R	209	78	48
20R	233	95	24
25R	224	87	24
26R	159	146	24

rum (Shahin & Spivey, 1986). Somaclones with alterations in karyotype, morphological and agronomical traits were found for *S. tuberosum* (Shepard *et al.*, 1980; Sidorov *et al.*, 1985). In the present investigation, protoclones of *S. rickii* and *S. lycopersicoides* with alterations in leaf morphology and ploidy levels were found. However it was impossible to examine the sexual progeny of these somaclones due to poor flowering. Somaclones of *S. rickii* in contrast to the parent did not flower. Both protoclones and the parent of *S. lycopersicoides* did not form flower buds, even after a year of vegetative growth in greenhouse.

Conclusions

The data reported here demonstrate successful isolation and cultivation of protoplasts for four species of series *Juglandifolia*. Plant regeneration and production of somaclones was achieved for two of these species, *S. rickii* and *S. lycopersicoides* is also possible. Based on the high regeneration potential of studied genotypes of these two species (Kut & Evans, 1982; Handley & Sink, 1985a, b; Tan *et al.*, 1987 and the results of our present investigation) one may suppose that within series *Juglandifolia* the plants of *lycopersicoides-rickii* group differ from *juglandifolium-ochranthum* ones not only by morphological but by physiological characteristics, in particular by totipotency level. Nevertheless, additional germplasm may need to be screened of *S. juglandifolium* and *S. ochranthum* to identify plants with better tissue culture competencies. Otherwise, for somatic hybridization between *S. ochranthum* or *S. juglandifolium* and cultivated tomato it may be necessary to use genotypes of *L. esculentum* possessing high regeneration ability.

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